Protocol

Analysis of Cell Viability by the Lactate Dehydrogenase Assay

Priti Kumar, Arvindhan Nagarajan, and Pradeep D. Uchil

A common method for determining cytotoxicity is based on measuring the activity of cytoplasmic enzymes released by damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is found in all cells. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage. LDH activity can be easily quantified by using the NADH produced during the conversion of lactate to pyruvate to reduce a second compound in a coupled reaction into a product with properties that are easily quantitated. This protocol measures the reduction of a yellow tetrazolium salt, INT, by NADH into a red, water-soluble formazan-class dye by absorbance at 492 nm. The amount of formazan is directly proportional to the amount of LDH in the culture, which is, in turn, directly proportional to the number of dead or damaged cells.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Cultured mammalian cells that have been transfected

The assay must be performed in triplicates. For an LDH assay, a set of wells with cells not exposed to the toxic agent needs to be set aside for estimating the total amount of LDH.

See Box 1 for important factors affecting assay quality.

LDH assay substrate solution <R>

LDH standards (optional; see Step 8)

Purified LDH is available commercially. Prepare a standard solution by dissolving LDH in cell culture medium, and dilute it to desired concentrations of 0.2–2.0 U/mL.

Lysis solution (9% [v/v] Triton X-100, for measurement of total cellular LDH)

Stop solution (50% dimethylformamide and 20% SDS at pH 4.7)

Hydrochloric acid (1 N) can be used to stop the reaction, but DMF/SDS solution is better when used with medium containing Phenol Red, because it neutralizes background absorption.

Equipment

Microtiter-plate spectrophotometer

Plates (96 well), standard flat-bottom wells

Plates (96 well), either V or round bottom

From the Molecular Cloning collection, edited by Michael R. Green and Joseph Sambrook.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot095497

BOX 1. IMPORTANT CONSIDERATIONS

Two factors in the cell culture medium can influence the background of LDH assays—Phenol Red and serum. The absorbance value of a culture medium control is used to normalize the values obtained from other samples. Background absorbance from Phenol Red can also be eliminated by using a Phenol Red-free medium. Serum contains a significant level of LDH activity. Human AB serum is relatively low in LDH activity, whereas calf serum is relatively high. Heat-inactivated serum has a much lower concentration of active LDH. In general, decreasing the serum concentration to 5% will significantly reduce background without affecting cell viability. Certain detergents (e.g., SDS and cetrimide) can inhibit LDH activity.

When performing the cytotoxicity assay, seed additional wells to perform the following controls.

1. *Spontaneous LDH release*. This corrects for spontaneous release of LDH from cells. Seed wells with cells that have not been transfected.
2. *Culture medium background*. This corrects for LDH activity contributed by serum in culture medium and the varying amounts of Phenol Red in the culture medium. Seed wells with culture medium alone.
3. *Maximum LDH release*. This yields an estimate of the maximum LDH activity that could be expected if all cells in the well were killed under the assay conditions used. For this, wells with untreated target cells are lysed with lysis solution and measured as described in Steps 1–8.

METHOD

Optimization of Target Cell Number (Total LDH Release Assay)

Because different target cell types (YAC-1, K562, Daudi, etc.) contain different amounts of LDH, it is valuable to perform a preliminary experiment using the cell type under investigation to determine the optimum number of target cells needed to ensure an adequate signal-to-noise ratio.

1. Prepare target cell dilutions (0, 5000, 10,000, and 20,000 cells/100 µL), and add 100 µL to each well in a V- or round-bottom, 96-well plate. Perform the assay in triplicate.
2. Add 15 µL of lysis solution to each well. Centrifuge the plate at 250*g* for 4 min.
3. Transfer 50 µL of supernatant to a 96-well, flat-bottom enzymatic assay plate.
4. Add 50 µL of LDH assay substrate to the medium. Cover the plate with foil or a small opaque box to protect it from light. Incubate for 15–30 min at 37°C.

   To include measurements for the preparation of a standard curve, see Step 8.
5. Add 100 µL of Stop solution.
6. Ensure that there are no bubbles in the wells. Measure the absorbance at 490 nm within 1 h of adding the Stop solution. Set the background absorbance at 690 nm, and subtract this value from the primary wavelength measurement (490 nm).
7. Determine the concentration of target cells yielding absorbance values at least two times the background absorbance of the medium control.
8. (Optional) Add 50 µL of assay substrate to 50 µL of different LDH standards. Incubate for 15 min and measure absorbance as above. A standard curve is prepared with the values obtained and is used to compare the enzyme activity of test samples.

Cytotoxicity Assay

9. Transfer 50 µL of cell culture supernatant into a 96-well plate. With suspension cell cultures, centrifuge the cells at 250*g* for 4 min, and then transfer 50 µL of the supernatant.

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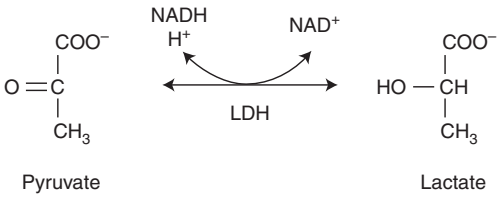

FIGURE 1. The reversible reaction catalyzed by lactate dehydrogenase.

10. Add 50 µL of LDH assay substrate to the medium. Cover the plate with foil or a small opaque box to protect it from light. Incubate it for 15–30 min at 37°C.
11. Add 100 µL of Stop solution.
12. Ensure that there are no bubbles in the wells. Measure the absorbance at 490 nm within 1 h of adding the Stop solution. Set the background absorbance at 690 nm, and subtract this value from the primary wavelength measurement (490 nm).
13. Determine the percent of cell death (% cytotoxicity) using the following equation:

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental LDH release } (OD_{490})}{\text{Maximum LDH release } (OD_{490})}.$$

14. (Optional) Add 50 µL of assay substrate to 50 µL of different LDH standards. Incubate for 15 min and measure the absorbance as above. A standard curve is prepared with the values obtained and is used to compare the enzyme activity of test samples.

DISCUSSION

There are five different isoforms of lactate dehydrogenase present in different tissue types, and they differ in their quantity, specificity, and kinetics of enzyme action. But all of the different enzyme isoforms catalyze the common reaction of interconverting pyruvate to lactate accompanied by the oxidation of NADH to NAD^+ (Fig. 1). The total LDH release assay provides a measure of the lactate dehydrogenase activity present in the cytoplasm of intact cells. Cell numbers can therefore be quantitated when the cells are lysed to release the LDH present inside. The number of cells present will be directly proportional to the absorbance values measured at 490 nm, which represent total LDH activity. The resulting data can be plotted with absorbance at 490-nm values along the ordinate and cell number along the abscissa.

Several companies market kits that use INT (2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride) as a substrate. Promega markets a kit that uses resazurin as a substrate for the coupled LDH assay, which can be measured as described in Protocol: **Analysis of Cell Viability by the alamarBlue Assay** (Kumar et al. 2018).

RECIPES

INT Solution

Dissolve 2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride (INT) in PBS to a final concentration of 100 mM.

LDH Assay Substrate Solution

L-(+)-lactic acid	0.054 M
β-NAD^+	1.3 mM
2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride (INT) solution <R>	0.66 mM
1-methoxyphenazine methosulfate (MPMS) solution <R>	0.28 mM
Tris–HCl buffer (pH 8.2)	0.2 M

Dissolve the ingredients in 0.2 M Tris–HCl buffer (pH 8.2). One milliliter of assay substrate is needed for 20 reactions.

Over time, competitive inhibitors of LDH can form in phosphate solutions of NADH. Prepare the LDH assay substrate solution fresh from stock solutions each time.

MPMS Solution

Dissolve 1-methoxyphenazine methosulfate (MPMS) in PBS to a concentration of 100 mM. Store for up to 1 mo at 4°C.

REFERENCES

Kumar P, Nagarajan A, Uchil PD. 2018. Analysis of cell viability by the alamarBlue assay. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot095489.

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